Comparative Silver Staining of Molluscan Spermatozoa

Mário SOUSA, *Elsa* OLIVEIRA, *João* CARVALHEIRO & *Víctor* OLIVEIRA

Laboratory of Cell Biology,
Institute of Biomedical Sciences, University of Oporto, Lg. Prof. Abel Salazar 2, 4050 Porto, Portugal

ABSTRACT

Mollusc spermatozoa were studied by transmission electron microscopy with the application of the silver nitrate method. Silver stained some components of the acrosomal vesicle and of the cytoskeletal elements of these cells in a pattern specific for each species. The importance of this staining method in relation to phylogenetic relationships is presented and discussed.

RÉSUMÉ

Coloration comparée à l'argent des spermatozoïdes de Mollusques

Des spermatozoïdes de Mollusques ont été étudiés en microscopie électronique à transmission grâce à la méthode au nitrate d'argent. Dans chaque espèce, l'argent colore certains composants de la vésicule acrosomienne et les éléments cytoplasmiques de ces cellules d'une manière spécifique. L'importance de cette méthode de coloration pour les relations phylogéniques est présentée et discutée.

The ultrastructural characteristics of mature spermatozoa have been used to examine phylogenetic relationships and have also been related to the different aspects of reproductive biology of the organism [18, 38, 39, 50, 51, 54]. In this respect, a great deal of work has been done on the ultrastructure of spermatozoa in the Patellogastropoda and Archaeogastropoda (Prosobranchia) as well as in the Heterodonta and Pteriomorphia (Bivalvia) [1-3, 6-37, 39-53, 55, 56, 59, 61-66].

In recent years, we have attempted to show that the silver staining method can be a useful and easy way to better understand specific aspects of spermatogenesis [57, 59, 60], as well as for comparative phylogenetical studies [58, 61-64]. In the present study, we present and critically review the silver staining characteristics of mature molluscan spermatozoa, with emphasis on its importance as a tool for studying phylogenetic relationships. We also provide the detailed technique so as to enable other laboratories to reproduce this staining method.

SOUSA, M., OLIVEIRA, E., CARVALHEIRO, J., & OLIVEIRA, V., 1995. — Comparative silver staining of molluscan spermatozoa. *In*: JAMIESON, B. G. M., AUSIO, J., & JUSTINE, J.-L. (eds), Advances in Spermatozoal Phylogeny and Taxonomy. *Mém. Mus. natn. Hist. nat.*, **166** : 179-187. Paris ISBN : 2-85653-225-X.

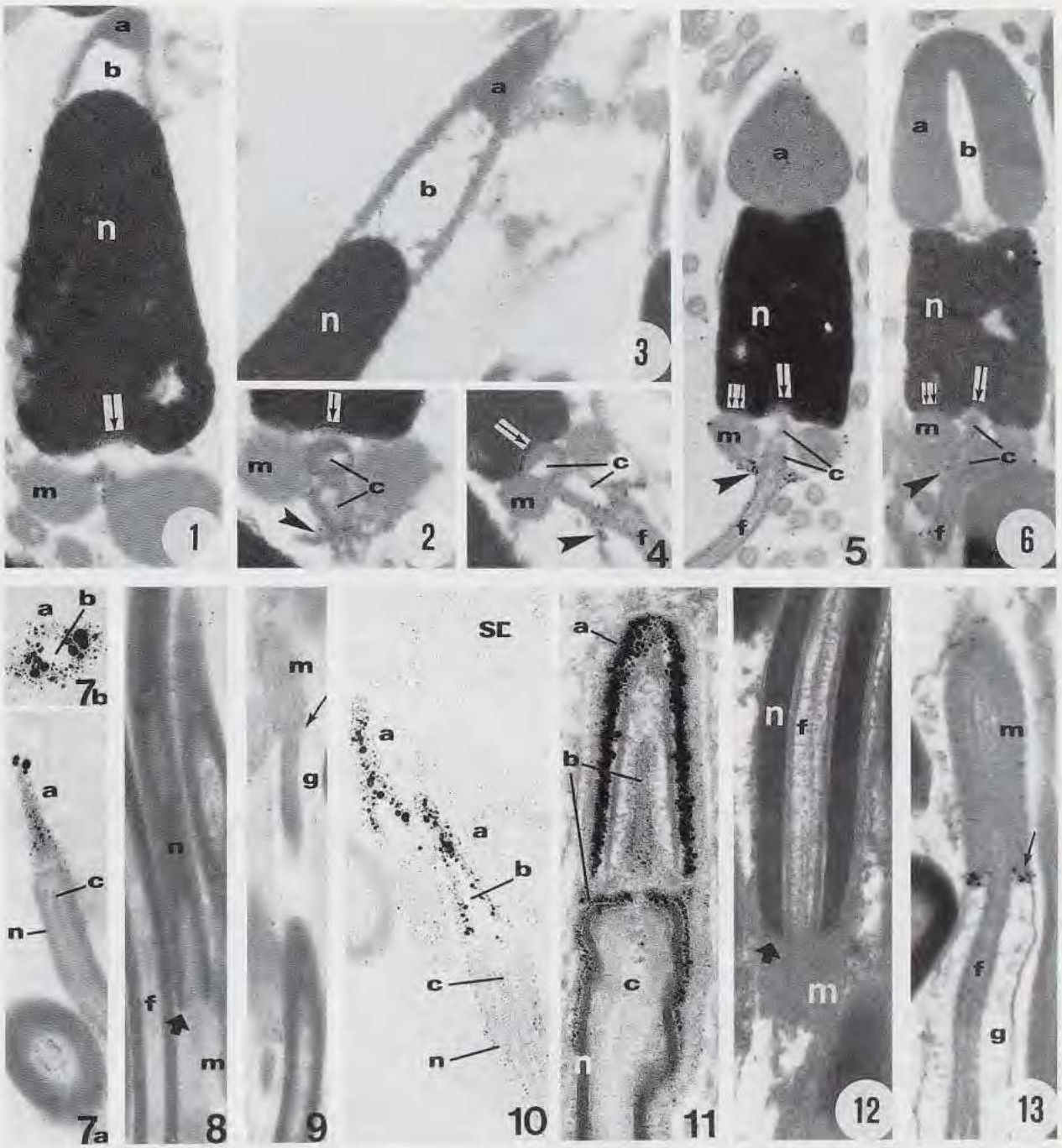
MATERIALS AND METHODS

Specimens (Table 1) were collected from sands at the Atlantic coast of Portugal and Spain. Small pieces of testis (< 1 mm) were fixed with 2.5% glutaraldehyde buffered with 0.2 M Na Cacodylate, pH 7.4, for 2 h at 4°C, rinsed in the same buffer for 2 h at 4°C, postfixed in Carnoy (acetic acid:methanol, 1:3) for 5 min at room temperature, gradually rehydrated in ethanol (10 min in each step), and rinsed in distilled water for 30 min. They were then immersed in 50% aqueous silver nitrate (filtered by 0.2 µm) and placed in a water-bath under a Philips lamp (500 W) in such a way as to reach 55°C for 10 min. Finally, the specimens were rinsed in distilled water (3 x 2 min), incubated in an ammoniacal silver-formalin solution (2 g silver nitrate in 2.5 ml distilled water + 2.5 ml 33% ammonia solution + 5 ml 3% aqueous formalin, 0.2 µm filtered) for 3 min, rinsed in distilled water (3 x 2 min), dehydrated in an ethanol graded series, embedded in Epon, and examined by transmission electron microscopy.

TABLE 1. — Silver staining of mature spermatozoa in the Mollusca.
AV, acrosomal vesicle; SAM, subacrosomal material; CF/MF, centriolar fossa/mitochondrial fossa; PCS, material that links the proximal centriole to the nuclear base; Ce, centrioles; PCC/An, pericentriolar complex/annulus.

Taxa	AV	SAM	CF/MF	PCS	Ce	PCC/An	Reference
Gastropoda							
Patellogastropoda							
Helcion pellucidus	-	-	+	-	±	±	[63]
Patella rustica	-	-	+	-	±	+	[63]
Archaeogastropoda							
Gibbula umbilicalis	+	-	+	-	±	+	present study
Gibbula cineraria	-	-	±	-	±	±	present study
Caenogastropoda							
Littorina saxatilis	+	-	-	-	-	-	[58]
Littorina littorea	+	-	-	-	+	-	[58]
Nucella lapillus	+	+	+	-	-	+	[4, 5]
Bivalvia							
Heterodonta							
Veneroida							
Donax trunculus	+	-	+	+	+	+	[61]
Spisula solidissima	+	+	+	-	±	+	[61]
Cerastoderma edule	+	-	+	+	-	-	[58]
Scrobicularia plana	-	-	+	-	-	+	[61]
Pteriomorphia							
Mytiloidea							
Mytilus edulis	+	-	-	-	-	-	[58]
Ostreoidea							
Ostrea edulis	+	-	+	+	-	-	[62]
Crassostrea angulata	+	+	+	+	+	+	[62]

FIGS 1-13. — Prosobranchia. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; f, flagellum; g, glycogen sheath. **1, 2:** *Helcion pellucidus*. Silver stains the centriolar fossa (arrow), and lightly stains the centrioles (c) and the pericentriolar complex (arrowhead). x 29 000. **3, 4:** *Patella rustica*. Silver stains the centriolar fossa (arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 24 600. **5:** *Gibbula umbilicalis*. Silver stains the acrosomal vesicle (a), the centriolar fossa (arrow), the mitochondrial fossa (double arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 17 900. **6:** *Gibbula cineraria*. Silver lightly stains the centriolar fossa (arrow), the mitochondrial fossa (double arrow), the centrioles (c), and the pericentriolar complex (arrowhead). x 17 900. **7-9:** *Littorina saxatilis*.



Silver stains the acrosomal vesicle (a). Large arrow, mitochondrial fossa; small arrow, annulus. x 32 000; x 18 600; x 18 600. **10:** *Littorina littorea*. Silver stains the acrosomal vesicle (a) and the centriole (c). Results for the mitochondrial fossa and the annulus are as for *L. saxatilis*. SC, sertoli cell. x 41 000. **11-13:** *Nucella lapillus*. Silver stains the acrosomal vesicle (a), the subacrosomal material (b), the mitochondrial fossa (large arrow), and the annulus (small arrow). x 51 600; x 30 000; x 33 000.

RESULTS AND DISCUSSION

Silver staining has been used to demonstrate the argyrophilia of acidic phosphoproteins contained in nucleolar components and their metaphase counterparts [60]. However, silver staining has also been shown to stain, in invertebrate species, other nuclear and cytoplasmic structures of germ cells and thereafter used to show different staining characteristics in different sperm species [57-64].

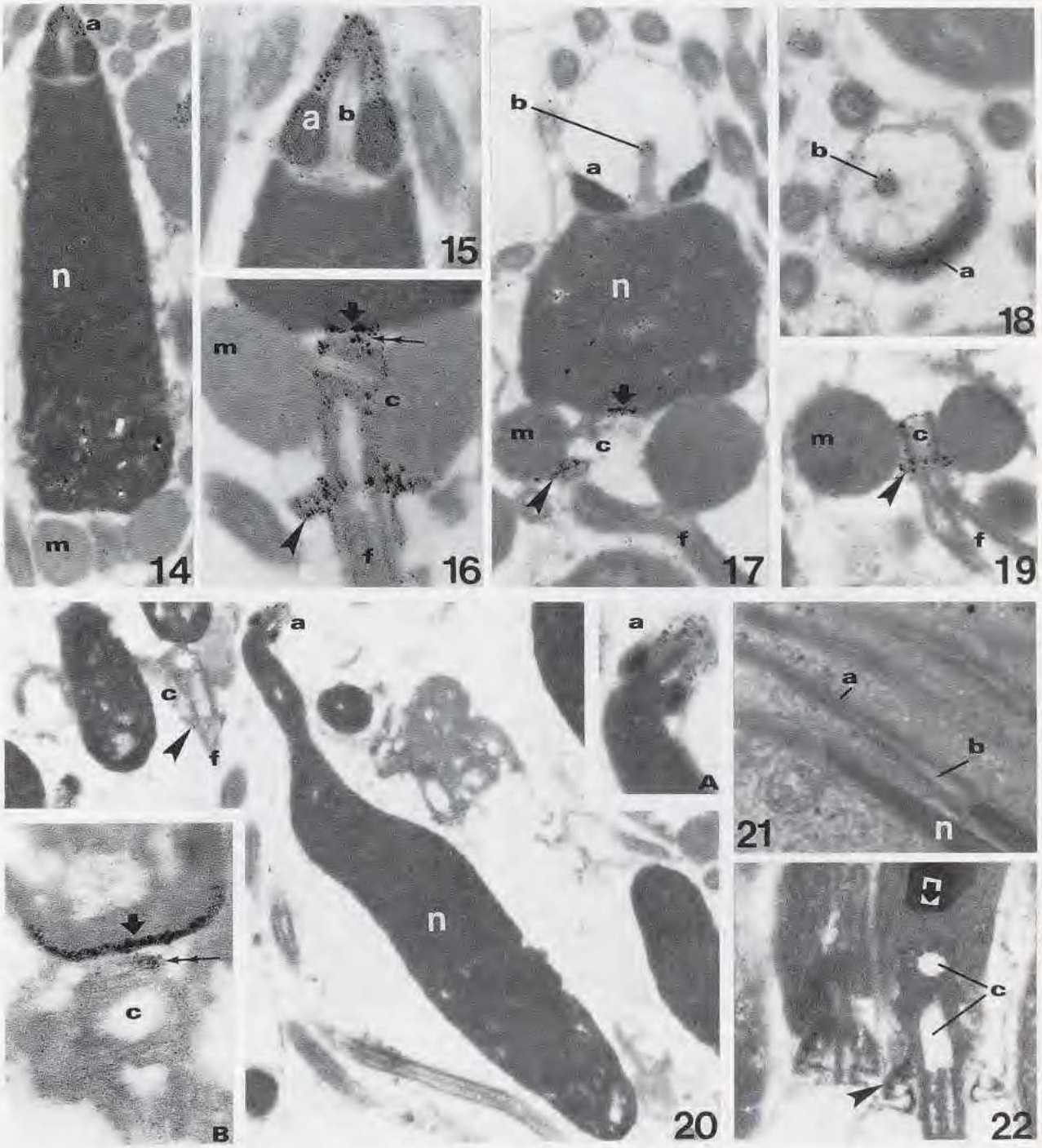
In the present work, we update all results obtained in the Mollusca with this silver staining technique, and discuss its relative importance as a phylogenetical sperm marker (Table 1). In the Patellogastropoda, *Helcion pellucidus* and *Patella rustica*, which are morphologically different, exhibit a similar pattern of silver staining (Figs 1-4); in the Archaeogastropoda, *Gibbula umbilicalis* and *G. cineraria* differ in nuclear and acrosomal vesicle dimensions as also in their silver staining characteristics (Figs 5, 6); in the Caenogastropoda, despite the morphological similarity between *Littorina saxatilis*, *L. littorea* and *Nucella lapillus*, their silver staining characteristics are very dissimilar (Figs 7-13). In the Veneroida, *Donax trunculus*, *Spisula solidissima*, *Cerastoderma edulis* and *Scrobicularia plana* are all very different in their morphological aspects, and also present very distinctive silver staining characteristics (Figs 14-22). In the Mytiloidea (Pteriomorphia), only *Mytilus edulis* has been studied by silver staining (Figs 23, 24); but in the Ostreoidea, despite the morphological similarity between *Ostrea edulis* and *Crassostrea angulata*, both species can be easily and completely differentiated by silver staining (Figs 25-27).

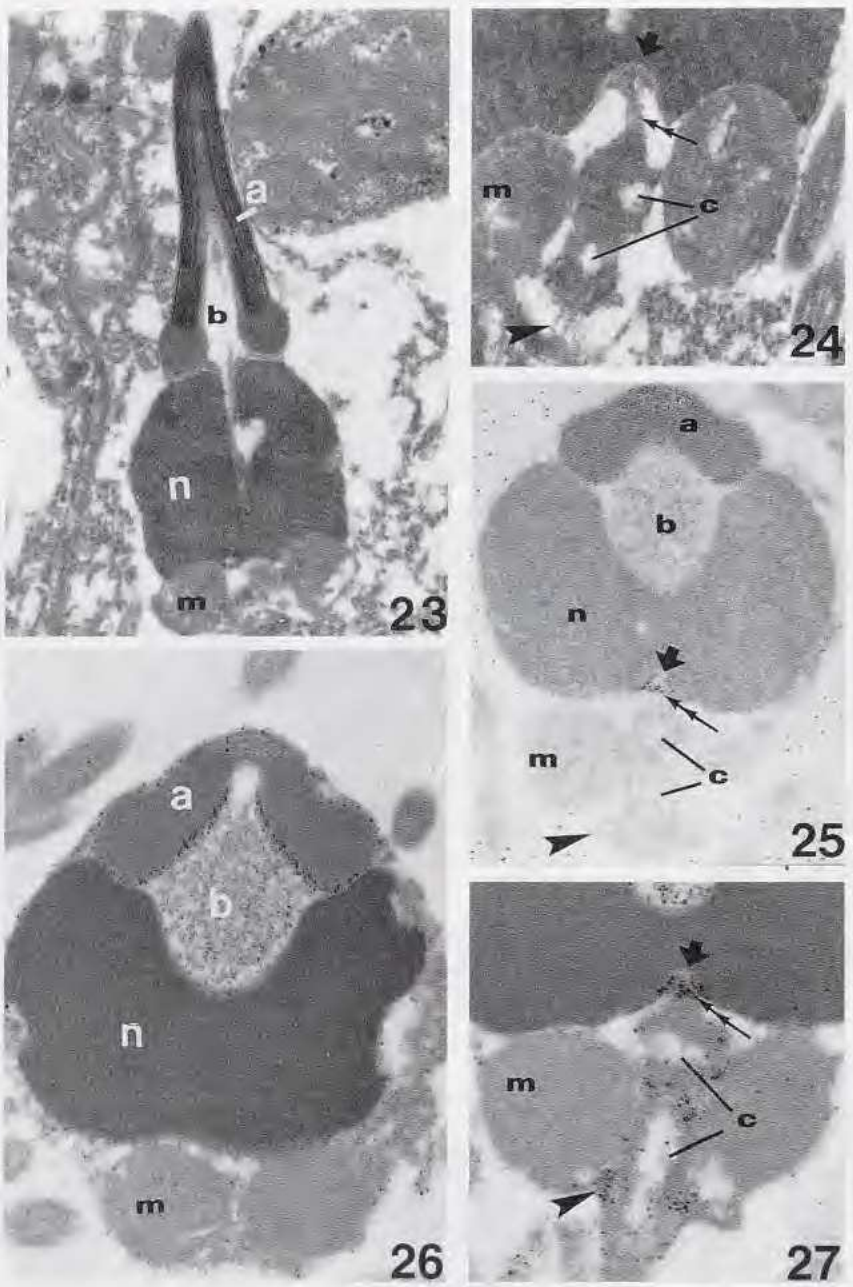
In conclusion, the silver staining of mature spermatozoa can enable us to distinguish different sperm species which present identical ultrastructural characteristics, and it can also help in phylogenetical studies since spermatozoa of the same group frequently share several silver staining characteristics (Table 1). However, the validation of this cytochemical marker as a useful tool in phylogenetical studies awaits further studies on many other molluscan species.

ACKNOWLEDGEMENTS

This work was supported by JNICT, the Arts and Sciences Institute, and the Eng. António de ALMEIDA Foundation. We would like to thank Dr. John HEALY for critically reviewing the manuscript.

FIGS 14-22. — Veneroidea. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; f, flagellum. **14-16:** *Donax trunculus*. Silver stains the acrosomal vesicle (a), the centriolar fossa (arrow), the material that links the proximal centriole to the centriolar fossa (double arrow), the centrioles (c), and the pericentriolar complex (arrowhead). x 22 500; x 40 000; x 40 000. **17-19:** *Spisula solidissima*. Silver stains the dense component of the acrosomal vesicle (a), the insertion of the tip of the perforatorium into the acrosomal vesicle (b), the centriolar fossa (arrow) and the pericentriolar complex (arrowhead), and lightly stains the centrioles (c). x 25 700; x 36 200; x 27 600. **20:** *Cerastoderma edule*. Silver stains the acrosomal vesicle (a), the nuclear base (arrow), and the material that links the proximal centriole to the nuclear base (double arrows). Arrowhead, pericentriolar complex. x 20 000; inset A, x 46 000; inset B, x 80 000. **21, 22:** *Scrobicularia plana*. Silver stains the nuclear base (arrow) and the pericentriolar complex (arrowhead). x 16 900; x 47 400.





FIGS 23-27. — Pteriomorphia. a, acrosomal vesicle; b, subacrosomal material; n, nucleus; m, mitochondria; c, centrioles; large arrow, centriolar fossa; double arrows, material that links the proximal centriole to the centriolar fossa; arrowhead, pericentriolar complex. **23, 24:** *Mytilus edulis*. Silver stains the acrosomal vesicle. x 17 600; x 40 700. **25:** *Ostrea edulis*. Silver stains the tip of the acrosomal vesicle (a), the centriolar fossa (large arrow), and the material that links the proximal centriole to the centriolar fossa (double arrows). x 28 900. **26, 27:** *Crassostrea angulata*. Silver stains the tip of the acrosomal vesicle (a), the subacrosomal material (b), the centriolar fossa (large arrow), the material that links the proximal centriole to the centriolar fossa (double arrows), the centrioles (c), and the pericentriolar complex (arrowhead). x 30 900; x 39 900.

REFERENCES

1. AL-HAJJ, H. A., 1988. — Ultrastructural analysis of sperm head development in *Nerita polita* (Mollusca: Archaeogastropoda) from the Jordan Gulf of Aqaba (Red Sea). *Invertebrate Reproduction and Development*, **13**: 281-295.
2. AL-HAJJ, H. A., 1990. — The fine structure of spermiogenesis and the mature spermatozoon of *Brachidontes variabilis* (Bivalvia). *Arabian Gulf Journal Scientific Research*, **8**: 153-162.
3. AZEVEDO, C., 1981. — The fine structure of the spermatozoon of *Patella lusitanica* (Gastropoda: Prosobranchia), with special reference to acrosome formation. *Journal of Submicroscopic Cytology*, **13**: 47-56.
4. AZEVEDO, C. & OLIVEIRA, E., 1988. — Presence of argyrophilic cytoplasmic and nuclear proteins in the spermatic cells of *Nucella lapillus* (Gastropoda, Prosobranchia). *Journal of Submicroscopic Cytology and Pathology*, **20**: 81-87.
5. AZEVEDO, C., CASTILHO, F. & BARANDELA, T., 1986. — Silver staining analysis on spermatozoa of *Nucella lapillus* (Gastropoda, Prosobranchia). *International Journal of Invertebrate Reproduction and Development*, **9**: 299-307.
6. AZEVEDO, C., LOBO-DA-CUNHA, A. & OLIVEIRA, E., 1985. — Ultrastructure of the spermatozoon in *Gibbula umbilicalis* (Gastropoda, Prosobranchia), with special reference to acrosomal formation. *Journal of Submicroscopic Cytology*, **17**: 609-614.
7. BERNARD, R. T. F. & HODGSON, A. N., 1985. — The fine structure of the sperm and spermatid differentiation in the brown mussel *Perna perna*. *South African Journal of Zoology*, **20**: 5-9.
8. BERNARD, R. T. F., DAVIES, B. R. & HODGSON, A. N., 1988. — Reproduction in a brackish-water mytilid: gametogenesis and embryonic development. *Veliger*, **30**: 278-290.
9. BOUCART, C., LAVALLARD, R. & LUBET, P., 1965. — Ultrastructure du spermatozoïde de la moule (*Mytilus perna* von Ihering). *Comptes Rendus de l'Académie des Sciences, Paris*, **260**: 5096-5099.
10. BOZZO, M. G., RIBES, E., SAGRISTA, E., POQUET, M. & DURFORT, M., 1993. — Fine structure of the spermatozoa of *Crassostrea gigas* (Mollusca, Bivalvia). *Molecular Reproduction and Development*, **34**: 206-211.
11. BUCKLAND-NICKS, J. & CHIA, F.-S., 1986. — Formation of the acrosome and basal body during spermiogenesis in a marine snail, *Nerita picea* (Mollusca: Archaeogastropoda). *Gamete Research*, **15**: 13-23.
12. BUCKLAND-NICKS, J. & CHIA, F.-S., 1986. — Fine structure of Sertoli cells in three marine snails with a discussion on the functional morphology of Sertoli cells in general. *Cell and Tissue Research*, **245**: 305-313.
13. CASAS, M. T. & SUBIRANA, J. A., 1994. — Chromatin condensation and acrosome development during spermiogenesis of *Ensis ensis* (Mollusca, Bivalvia). *Molecular Reproduction and Development*, **37**: 223-228.
14. DANIELS, E. W., LONGWELL, A. C., MCNIFF, J. M. & WOLFGANG, R. W., 1971. — Ultrastructure of spermatozoa from the american oyster *Crassostrea virginica*. *Transactions of the American Microscopical Society*, **90**: 275-282.
15. DORANGE, G. & LE PENNEC, M., 1989. — Ultrastructural characteristics of spermatogenesis in *Pecten maximus* (Mollusca, Bivalvia). *Invertebrate Reproduction and Development*, **15**: 109-117.
16. ECKELBARGER, K. J., BIELER, R. & MIKKELSEN, P. M., 1990. — Ultrastructure of sperm development and mature sperm morphology in three species of commensal bivalves (Mollusca: Galeommatoidea). *Journal of Morphology*, **205**: 63-75.
17. ENDO, S., 1976. — Silver methenamine and phosphotungstic acid staining of the acrosome of *Mytilus edulis*. *Experimental Cell Research*, **100**: 71-78.
18. FRANZÉN, A., 1983. — Ultrastructural studies of spermatozoa in three bivalve species with notes on evolution of elongated sperm nucleus in primitive spermatozoa. *Gamete Research*, **7**: 199-214.
19. GARREAU DE LOUBRESSE, N., 1971. — Spermiogenèse d'un gastéropode prosobranche: *Nerita senegalensis*; évolution du canal intranucléaire. *Journal de Microscopie*, **12**: 425-440.
20. GHARAGOZLOU, I. D., GINNEKEN, V. & POCHON-MASSON, J., 1971. — Étude comparative infrastructurale du spermatozoïde chez les palourdes de France. *Archives de Zoologie Expérimentale et Générale*, **112**: 805-817.
21. GIUSTI, F. & SELMI, M. G., 1982. — The morphological peculiarities of the typical spermatozoa of *Theodoxus fluviatilis* (L.) (Neritoidea) and their implications for motility. *Journal of Ultrastructure Research*, **78**: 166-177.
22. GUTIÉRREZ, M., CRESPO, J. P. & PASCUAL, E., 1978. — Ultraestructura de ovocitos y espermatozoides del ostion, *Crassostrea angulata* Lmk de la costa sudatlantica de Espana. *Investigaciones Pesqueras*, **42**: 167-178.
23. HEALY, J. M., 1988. — Ultrastructural observations on the spermatozoa of *Pleurotomaria africana* Tomlin (Gastropoda). *Journal of Molluscan Studies*, **54**: 309-316.
24. HEALY, J. M., 1989. — Ultrastructure of spermiogenesis in the gastropod *Calliotropis glyptus* Watson (Prosobranchia: Trochidae) with special reference to the embedded acrosome. *Gamete Research*, **24**: 9-19.

25. HEALY, J. M., 1990. — Sperm structure in the scissurellid gastropod *Sinezona* sp. (Prosobranchia, Pleurotomarioidea). *Zoologica Scripta*, **19**: 189-193.

26. HEALY, J. M., 1990. — Euspermatozoa and paraspermatozoa in the trochoid gastropod *Zalipais laseroni* (Trochoidea: Skeneidae). *Marine Biology*, **105**: 497-507.

27. HEALY, J. M. & LESTER, R. J. G., 1991. — Sperm ultrastructure in the australian oyster *Saccostrea commercialis* (Iredale & Roughley) (Bivalvia: Ostreoidea). *Journal of Molluscan Studies*, **57**: 219-224.

28. HODGSON, A. N., 1993. — Spermatozoon structure and spermiogenesis in *Nassarius kraussianus* (Gastropoda, Prosobranchia, Nassariinae). *Invertebrate Reproduction and Development*, **23**: 115-121.

29. HODGSON, A. N. & BERNARD, R. T. F., 1986. — Ultrastructure of the sperm and spermatogenesis of three species of Mytillidae (Mollusca, Bivalvia). *Gamete Research*, **15**: 123-135.

30. HODGSON, A. N. & BERNARD, R. T. F., 1988. — A comparison of the structure of the spermatozoa and spermatogenesis of 16 species of patellid limpet (Mollusca: Gastropoda: Archaeogastropoda). *Journal of Morphology*, **195**: 205-223.

31. HODGSON, A. N. & BERNARD, R. T. F., 1989. — Spermatozoon structure and the taxonomic affinity of the limpet *Nacella delesserti* (Gastropoda: Patellidae). *Journal of Molluscan Studies*, **55**: 145-147.

32. HODGSON, A. N. & CHIA, F.-S., 1993. — Spermatozoon structure of some North American prosobranchs from the families Lottiidae (Patellogastropoda) and Fissurellidae (Archaeogastropoda). *Marine Biology*, **116**: 97-101.

33. HODGSON, A. N. & FOSTER, G. G., 1992. — Structure of the sperm of some South African archaeogastropods (Mollusca) from the superfamilies Haliotoidea, Fissurelloidea and Trochoidea. *Marine Biology*, **113**: 89-97.

34. HODGSON, A. N., BERNARD, R. T. F. & VAN DER HORST, G., 1990. — Comparative spermatology of three species of *Donax* (Bivalvia) from South Africa. *Journal of Molluscan Studies*, **56**: 257-265.

35. HODGSON, A. N., DE VILLIERS, C. J. & BERNARD, R. T. F., 1987. — Comparative spermatology of two morphologically similar species of Solen (Mollusca: Bivalvia). *South African Journal of Zoology*, **22**: 264-268.

36. HODGSON, A. N., HELLER, J. & BERNARD, R. T. F., 1990. — Ultrastructure of the sperm and spermatogenesis in five South African species of the trochid genus *Oxystele* (Mollusca, Prosobranchia). *Molecular Reproduction and Development*, **25**: 263-271.

37. HYLANDER, B. L. & SUMMERS, R. G., 1977. — An ultrastructural analysis of the gametes and early fertilization in two bivalve molluscs, *Chama macerophylla* and *Spisula solidissima* with special reference to gamete binding. *Cell and Tissue Research*, **182**: 469-489.

38. JAMIESON, B. G. M., 1987. — A biological classification of sperm types, with special reference to Annelids and Molluscs, and an example of spermiocladistics. *In*: H. MOHRI, *New Horizons in Sperm Cell Research*. Tokyo, Japan Scientific Press/ New York, Gordon & Breach: 311-332.

39. JAMIESON, B. G. M., HODGSON, A. N. & BERNARD, R. T. F., 1991. — Phylogenetic trends and variation in the ultrastructure of the spermatozoa of sympatric species of South African patellid limpets (Archaeogastropoda, Mollusca). *Invertebrate Reproduction and Development*, **20**: 137-146.

40. KOHNERT, V. K. & STORCH, V., 1984. — Ultrastructure of sperm cells and spermiogenesis of *Neritina communis* (Quoy and Gaimard) (Mollusca, Gastropoda). *Zoologisches Anzeiger Jena*, **212**: 85-94.

41. LEWIS, C. A., LEIGHTON, D. L. & VACQUIER, V. D., 1980. — Morphology of abalone spermatozoa before and after the acrosome reaction. *Journal of Ultrastructure Research*, **72**: 39-46.

42. LONGO, F. J. & ANDERSON, E., 1969. — Spermiogenesis in the surf clam *Spisula solidissima* with special reference to the formation of the acrosomal vesicle. *Journal of Ultrastructure Research*, **27**: 435-443.

43. LONGO, F. J. & DORNFELD, E. J., 1967. — The fine structure of spermatid differentiation in the mussel, *Mytilus edulis*. *Journal of Ultrastructure Research*, **20**: 462-480.

44. NICOTRA, A. & ZAPPATA, S., 1991. — Ultrastructure of the mature sperm and spermiogenesis in *Callista chione* (Mollusca, Bivalvia). *Invertebrate Reproduction and Development*, **20**: 213-218.

45. Ó FOIGHIL, D., 1985. — Fine structure of *Lasaea subviridis* and *Mysella tumida* sperm (Bivalvia, Galeommatacea). *Zoomorphology*, **105**: 125-132.

46. Ó FOIGHIL, D., 1985. — Form, function, and origin of temporary dwarf males in *Pseudopythina rugifera* (Carpenter, 1864) (Bivalvia: Galeommatacea). *Veliger*, **27**: 245-252.

47. Ó FOIGHIL, D., 1989. — Role of spermatozeugmata in the spawning ecology of the brooding oyster *Ostrea edulis*. *Gamete Research*, **24**: 219-228.

48. PASTEELS, J. J. & DE HARVEN, E., 1962. — Étude au microscope électronique du spermatozoïde d'un mollusque bivalve, *Barnea candida*. *Archives de Biologie (Liège)*, **73**: 445-463.

49. POCHON-MASSON, J. & GHARAGOZLOU, I.-D., 1970. — Particularité morphologique de l'acrosome dans le spermatozoïde de *Tapes decussatus* L. (mollusque lamellibranche). *Annales des Sciences Naturelles, Zoologie*, **12**: 171-180.

50. POPHAM, J. D., 1974. — Comparative morphometrics of the acrosomes of the sperms of externally and internally fertilizing sperms of the shipworms (Teredinidae, Bivalvia, Mollusca). *Cell and Tissue Research*, **150**: 291-297.

51. POPHAM, J. D., 1979. — Comparative spermatozoon morphology and bivalve phylogeny. *Malacological Reviews*, **12**: 1-20.

52. POPHAM, J. D., DICKSON, M. R. & GODDARD, C. K., 1974. — Ultrastructural study of the mature gametes of two species of *Bankia* (Mollusca: Teredinidae). *Australian Journal of Zoology*, **22**: 1-12.

53. REUNOV, A. A. & HODGSON, A. N., 1994. — Ultrastructure of the spermatozoa of five species of South African bivalves (Mollusca), and an examination of early spermatogenesis. *Journal of Morphology*, **219**: 275-283.

54. ROUSE, G. W. & JAMIESON, B. G. M., 1987. — An ultrastructural study of the spermatozoa of the polychaetes *Eurythoe complanata* (Amphinomidae), *Clymenella* sp. and *Micromaldane* sp. (Maldanidae), with definition of sperm types in relation to reproductive biology. *Journal of Submicroscopic Cytology*, **19**: 573-584.

55. SELMI, M. G. & GIUSTI, F., 1983. — The atypical spermatozoon of *Theodoxus fluviatilis* (L.) (Gastropoda, Prosobranchia). *Journal of Ultrastructure Research*, **84**: 173-181.

56. SMALDON, P. R. & DUFFUS, J. H., 1985. — An ultrastructural study of gametes and fertilization in *Patella vulgata* L. *Journal of Molluscan Studies*, **51**: 116-132.

57. SOUSA, M. & AZEVEDO, C., 1988. — Ultrastructure and silver staining analysis of spermatogenesis in the sea urchin *Paracentrotus lividus* (Echinodermata, Echinoidea). *Journal of Morphology*, **195**: 177-188.

58. SOUSA, M. & AZEVEDO, C., 1988. — Comparative silver staining analysis on spermatozoa of various invertebrate species. *International Journal of Invertebrate Reproduction and Development*, **13**: 1-8.

59. SOUSA, M. & AZEVEDO, C., 1989. — Silver staining of spermatogenesis in the starfish *Marthasterias glacialis*. *Invertebrate Reproduction and Development*, **15**: 105-108.

60. SOUSA, M. & AZEVEDO, C., 1990. — Ultrastructure and silver staining of echinoderm spermatogenesis. *In*: B. DALE, *Mechanism of Fertilization: Plants to Human*. NATO ASI Series, Vol. H45. Berlin/Heidelberg, Springer-Verlag: 115-129.

61. SOUSA, M. & OLIVEIRA, E., 1994. — Ultrastructural and cytochemical study of spermatogenesis in *Donax trunculus* (Mollusca, Bivalvia). *Journal of Submicroscopic Cytology and Pathology*, **26**: 305-311.

62. SOUSA, M. & OLIVEIRA, E., 1994. — An ultrastructural study of spermatogenesis in *Crassostrea angulata* (Mollusca, Bivalvia). *Marine Biology*, **120**: 545-551.

63. SOUSA, M. & OLIVEIRA, E., 1994. — An ultrastructural study of spermatogenesis of *Helcion pellucidus* (Gastropoda, Prosobranchia). *Invertebrate Reproduction and Development*, **26**: 119-126.

64. SOUSA, M., CORRAL, L. & AZEVEDO, C., 1989. — Ultrastructural and cytochemical study of spermatogenesis in *Scrobicularia plana* (Mollusca, Bivalvia). *Gamete Research*, **24**: 393-401.

65. THIELLEY, M., WEPPE, M. & HERBAUT, C., 1993. — Ultrastructural study of gametogenesis in the french polynesian black pearl oyster *Pinctada margaritifera* (Mollusca, Bivalvia). I. Spermatogenesis. *Journal of Shellfish Research*, **12**: 41-47.

66. VAN DER HORST, G., HODGSON, A. N., BERNARD, R., SALIE, W. & MAASDORP, I. J. W., 1986. — Sperm ultrastructure of two *Donax* (Bivalvia) species. *Proceedings of the Electron Microscopy Society of South Africa*, **16**: 69-70.